Vertical Profiles Of Methanogenesis And Methanogens In Two Contrasting Acidic Peatlands In Central New York State, USA

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Abstract

Northern acidic peatlands are important sources of atmospheric methane, yet the methanogens in them are poorly characterized. We examined methanogenic activities and methanogen populations at different depths in two peatlands, McLean bog (MB) and Chicago bog (CB). Both have acidic (pH 3.5–4.5) peat soils, but the pH of the deeper layers of CB is near-neutral, reflecting its previous existence as a neutral-pH fen. Acetotrophic and hydrogenotrophic methanogenesis could be stimulated in upper samples from both bogs, and phylotypes of methanogens using H2/CO2 (Methanomicrobiales) or acetate (Methanosarci-nales) were identified in 16S rRNA gene clone libraries and by terminal restriction fragment length polymorphism (T-RFLP) analyses using a novel primer/restriction enzyme set that we developed. Particularly dominant in the upper layers was a clade in the Methanomicrobiales, called E2 here and the R10 or fen group elsewhere, estimated by quantitative polymerase chain reaction to be present at ~108 cells per gram of dry peat. Methanogenic activity was considerably lower in deeper samples from both bogs. The methanogen populations detected by T-RFLP in deeper portions of MB were mainly E2 and the uncultured euryarchaeal rice cluster (RC)-II group, whereas populations in the less acidic CB deep layers were considerably different, and included a Methanomicrobiales clade we call E1-E1¢, as well as RC-I, RC-II, marine benthic group D, and a new cluster that we call the subaqueous cluster. E2 was barely detectable in the deeper samples from CB, further evidence for the associations of most organisms in this group with acidic habitats.

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Introduction

Although peatlands in northern latitudes account for 5–10% of the methane emitted into the Earth's atmosphere (Whalen, 2005), little is known about the composition and abundance of the microorganisms responsible for methane production in these systems. A better understanding of these methanogens is needed to predict how emissions of atmospheric methane from northern peatlands will respond to global environmental changes (Wieder and Yavitt, 1994; Brown, 1998).

Northern peatlands include a wide range of ecosystems, each with a characteristic peat soil derived from partially decaying plant material and with little or no rock-derived minerals. At one end are the most acidic systems, called bogs, characterized by low mineral nutrient concentrations and dominated by *Sphagnum* mosses and a few ericaceous shrub species (Crum, 1992). Increasing pH is associated with less *Sphagnum* and dominance by *Carex* sedges and graminoid plants, resulting in near-neutral-pH systems, called fens (Crum, 1992). A characteristic of many northern peatlands is that the plant species composition of surface vegetation can be quite different from the remains of plants in the peat that dominated in the past (Barber, 1981).

Numerous methanogens have been cultivated and characterized from diverse habitats (Garcia, 1990), but initial attempts at cultivation of indigenous methanogens from bogs were unsuccessful (Williams and Crawford, 1985; Goodwin and Zeikus, 1987). However, the application of molecular techniques based on 16S rRNA and *mcrA* (encoding a subunit of the methylreductase involved in methanogenesis) gene sequences has revealed a diversity of methanogens as well as related *Euryarchaeota* in bogs (Hales *et al*., 1996; Basiliko *et al*., 2003; Kotsyurbenko *et al*., 2004; Galand *et al*., 2005). At this point in time, none of the sequences in acidic peat bogs match those of isolated methanogens, although related sequences have recently been detected in enrichment cultures (Horn *et al*., 2003; Sizova *et al*., 2003).

In this study, we examined the depth (vertical) distribution of methanogenic activity and methanogenic *Archaea* in two northern peatlands in New York State. One, known locally as 'Chicago bog' (CB) has acidic bog plants growing over what was once a neutral-pH fen so that lower layers are less acidic. The other site, known locally as

Table 1. Main physicochemical characteristics of peat along the vertical profiles from Chicago bog and McLean bog.

	pH		% dry weight	
Depth (cm)	Chicago bog	McLean bog	Chicago bog	McLean bog
10	3.92 ± 0.07	3.58 ± 0.42	5.42 ± 0.09	4.22 ± 1.08
15/20	4.06 ± 0.06	4.03 ± 0.06	5.25 ± 0.06	7.88 ± 0.21
40	4.87 ± 0.13	4.19 ± 0.02	12.12 ± 0.11	9.12 ± 0.4
65	5.71 ± 0.12	4.21 ± 0.04	11.91 ± 0.21	7.5 ± 0.23

Peat samples were taken at the indicated depths; mean ± SD; $n = 3$.

'McLean bog' (MB), was acidic throughout the profile. We demonstrate here that terminal restriction fragment length polymorphism (T-RFLP) analyses of methanogen populations of all samples from MB showed dominance of a clade in the *Methanomicrobiales* associated with acidic habitats, whereas methanogen populations in lower, more neutral, samples from CB differed considerably from the acidic upper layers.

Results

Physicochemical characteristics of vertical profiles of peat cores

Stratigraphy of the cores from both sites indicated a 10 cm cap of light brown coloured sphagnum peat underlain by a more degraded softer in consistency, dark brown peat at 15–20 cm. At depths below 40 cm, the peat was highly decomposed and darker in colour indicating highly reduced conditions. The peat in deeper layers (40 and 65 cm depth) of CB had decreasing amounts of *Sphagnum* remains and was herbaceously derived, i.e. mostly *Carex* rhizomes, whereas deeper layers in MB consisted mostly of sphagnum remains. The pH in deeper layers of MB remained low, but in CB it increased to 4.9 and 5.7, respectively, at 40 and 65 cm depths (Table 1). The dry matter content was greater at lower depths in both cores, with a greater increase in the CB core.

CH_4 production by core samples

Rates of CH_4 production from endogenous substrates in samples from the 40 cm depth were less than 15% of those in the 15–20 cm depth (Fig. 1), and even slower rates occurred in samples from 65 cm depth (data not presented). Added acetate had no effect on rates of CH_4 production until approximately 20 days of incubation when CH_4 production in the acetate-amended samples from upper layers of MB and the 15 cm layer of CB (Fig. 1A and B) increased relative to unamended samples. Added acetate did not stimulate CH_4 production in the 10 cm layer of CB or in the 40 cm (Fig. 1C) and 65 cm samples from either bog during the entire 63-day incubation time.

A previous report at the same study sites showed that the build-up of acetic acid inhibited CH_4 production in peat amended with H_2/CO_2, and that the addition of rifampicin avoided this interference by acetogenic organisms (Bräuer *et al*., 2004). CH_4 production in upper layers by samples amended with H_2/CO_2 and rifampicin was significantly greater than the rifampicin-only controls. Rates of methanogenesis increased during the first days of incubation for both sites (Fig. 1A and B), indicative of methanogen growth. In deeper layers, the CH_4 production in H_2/CO_2-amended samples started to increase above the unamended control only after more than 20–30 days of incubation (Fig. 1C). Samples from 65 cm presented similar, although slower, response compared with those from 40 cm (not shown).

16S rRNA phylogenetic diversity

In order to study methanogen community composition we used cloning and sequence analyses of the 16S rRNA gene. We utilized the 1AF-1100R set of primers (Hales *et al*., 1996) because our initial studies on clone libraries developed with different archaeal primer sets indicated a good coverage of methanogenic *Archaea* in our study sites (H. Cadillo-Quiroz, unpublished). Comparison of reported sequences for shallow layers of MB (Basiliko *et al*., 2003) and sequences from our clone libraries for CB shallow layers showed a similar phylogenetic composition of methanogenic archaeal groups (Fig. 2). Sequences were associated with *Methanosarcinaceae* and *Methanosaetaceae* families, and the *Methanomicrobiales*. Uncultured groups such us rice cluster (RC)-I and -II were present in both bogs. Clones with phylotypes associated with marine benthic group D (MBD) (Vetriani *et al*., 1999) were recovered only from the deeper layers of CB. Additionally, we observed a cluster of sequences that were not positioned within the other previously described groups. Those sequences formed a distinct cluster related mainly to environmental phylotypes retrieved from immersed or subaqueous environments like lake sediments, Siberian deep wells, deep-sea chimneys and a nearby neutral fen (J. Yavitt, unpublished). This cluster of sequences, which we have named the subaque-

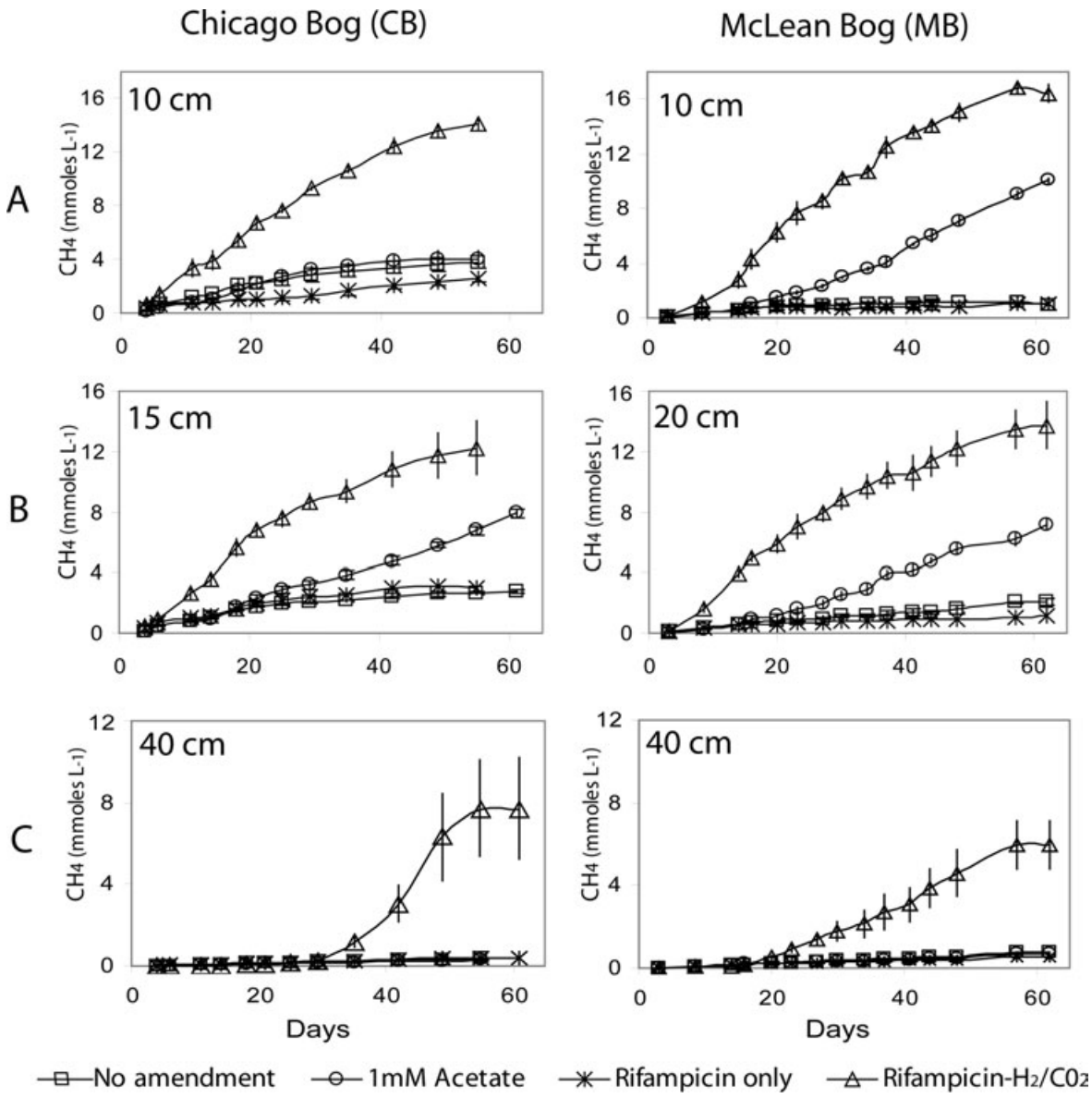


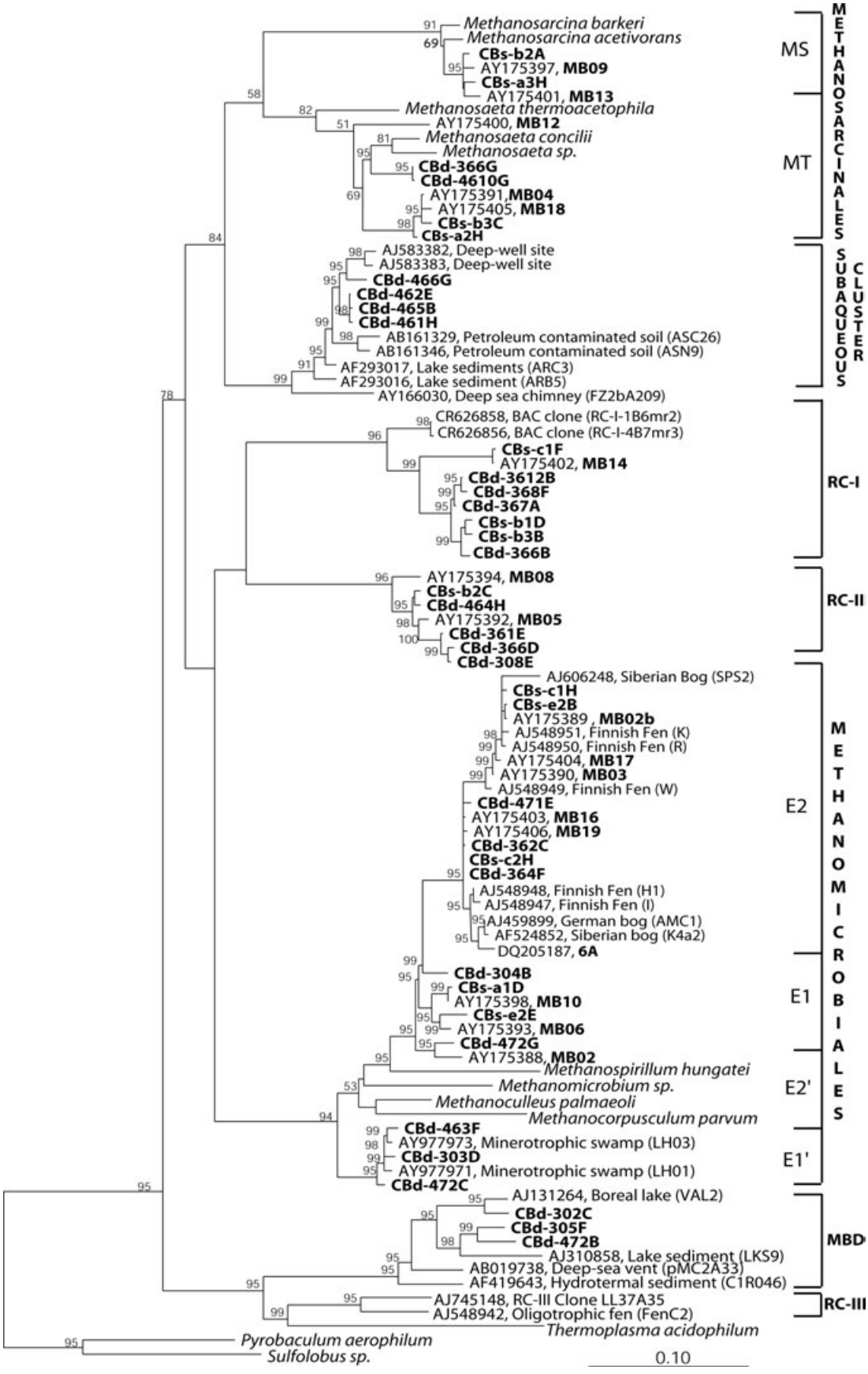
Fig. 1. Time-course for methane production by peat slurries from Chicago bog and McLean bog vertical profiles. (A) 10 cm deep, (B) 15/20 cm deep and (C) 40 cm deep incubated at approximately 23°C; mean ± SD; $n = 3$.

ous cluster (SC), represents a new group of uncharacterized euryarchaeota.

T-RFLP analysis of community structure

We used T-RFLP analysis to obtain a description of the dominant groups in the archaeal community accessible through our primer set. The 1Af-1100R set of primers have not been used previously in T-RFLP analysis, and in this study we defined T-RFLP parameters that minimized ambiguous identification. A T-RFLP protocol using two restriction enzymes (HhaI and Sau96I) was designed by

Fig. 2. Phylogenetic tree for archaeal 16S rRNA gene clones from McLean bog (MB) and Chicago bog (CB). *Methanosarcinales*: *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT); subaqueous cluster (SC); rice cluster-I (RC-I); rice cluster-II (RC-II); *Methanomicrobiales*: group E1, group E1′, group E2 and group E2′ (classification based on Basiliko *et al*., 2003); marine benthic group D (MBD) (based on Vetriani *et al*., 1999). Euryarchaeal and crenarchaeal nearly complete 16S rRNA gene sequences were used to construct tree with the neighbour joining and quartet PUZZLE methods. Tree topology was confirmed with maximum likelihood algorithm, and bootstrap values (100 trees) greater than 50 are indicated. GenBank accession numbers of added sequences are indicated. Clones from CB shallow and deeper layers are indicated by the initials CBs and CBd respectively. 6A correspond to methanogenic culture from MB (Bräuer *et al*., 2006).


Methanosarcina barkeri
Methanosarcina acetivorans
CBs-b2A
AY175397, MB09
CBs-a3H
AY175401, MB13
Methanosaeta thermoacetophila
AY175400, MB12
Methanosaeta concilii
Methanosaeta sp.
CBd-366G
CBd-4610G
AY175391,MB04
AY175405, MB18
CBs-b3C
CBs-a2H
AJ583382, Deep-well site
AJ583383, Deep-well site
CBd-466G
CBd-462E
CBd-465B
CBd-461H
AB161329, Petroleum contaminated soil (ASC26)
AB161346, Petroleum contaminated soil (ASN9)
AF293017, Lake sediments (ARC3)
AF293016, Lake sediment (ARB5)
AY166030, Deep sea chimney (FZ2bA209)
CR626858, BAC clone (RC-I-1B6mr2)
CR626856, BAC clone (RC-I-4B7mr3)
CBs-c1F
AY175402, MB14
CBd-3612B
CBd-368F
CBd-367A
CBs-b1D
CBs-b3B
CBd-366B
AY175394, MB08
CBs-b2C
CBd-464H
AY175392, MB05
CBd-361E
CBd-366D
CBd-308E
AJ606248, Siberian Bog (SPS2)
CBs-c1H
CBs-e2B
AY175389 , MB02b
AJ548951, Finnish Fen (K)
AJ548950, Finnish Fen (R)
AY175404, MB17
AY175390, MB03
AJ548949, Finnish Fen (W)
CBd-471E
AY175403, MB16
AY175406, MB19
CBd-362C
CBs-c2H
CBd-364F
AJ548948, Finnish Fen (H1)
AJ548947, Finnish Fen (I)
AJ459899, German bog (AMC1)
AF524852, Siberian bog (K4a2)
DQ205187, 6A
CBd-304B
CBs-a1D
AY175398, MB10
CBs-e2E
AY175393, MB06
CBd-472G
AY175388, MB02
Methanospirillum hungatei
Methanomicrobium sp.
Methanoculleus palmaeoli
Methanocorpusculum parvum
CBd-463F
AY977973, Minerotrophic swamp (LH03)
CBd-303D
AY977971, Minerotrophic swamp (LH01)
CBd-472C
AJ131264, Boreal lake (VAL2)
CBd-302C
CBd-305F
CBd-472B
AJ310858, Lake sediment (LKS9)
AB019738, Deep-sea vent (pMC2A33)
AF419643, Hydrotermal sediment (C1R046)
AJ745148, RC-III Clone LL37A35
AJ548942, Oligotrophic fen (FenC2)
Thermoplasma acidophilum
Pyrobaculum aerophilum
Sulfolobus sp.
0.10
MS
MT
METHANOSARCINALES
SUBAQUEOUS CLUSTER
RC-I
RC-II
E2
E1
E2'
E1'
METHANOMICROBIALES
MBD
RC-III

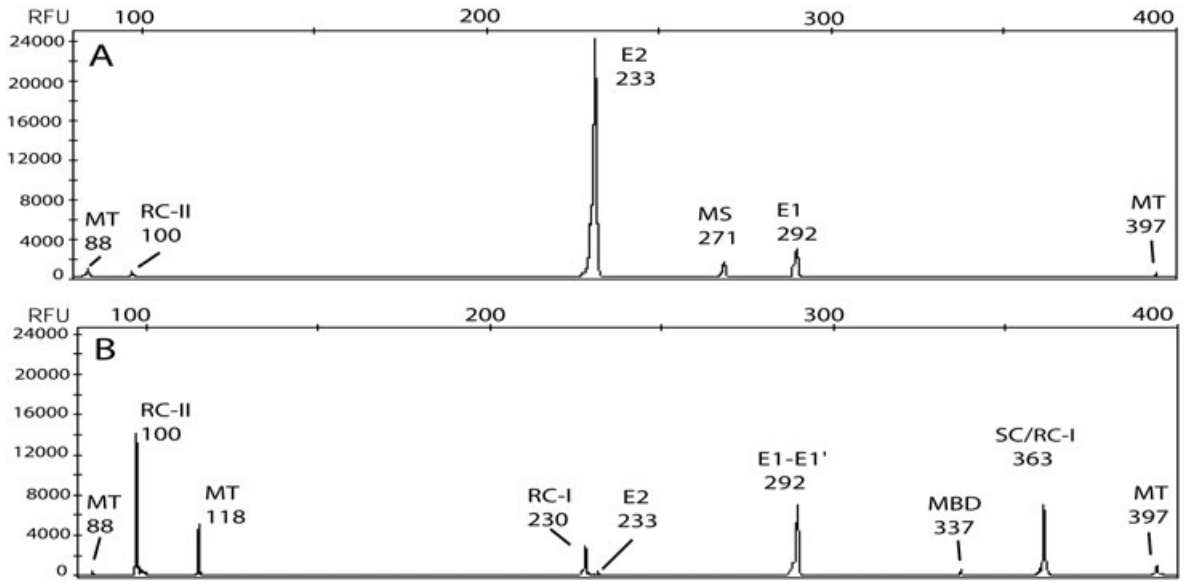


Fig. 3. Representative T-RFLP electropherograms of archaeal 16S rRNA gene fragments amplified with the 1AF-1100R (Hales *et al*., 1996) from DNA extracts from peat samples from McLean bog and Chicago bog. (A) McLean bog 20 cm deep, and (B) Chicago bog 40 cm deep. The *x*-axis and the numbers above the peaks show the length (bp) of the terminal restriction fragments (T-RFs). The archaeal groups represented by distinctive T-RFs are: *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT), rice cluster-I (RC-I), rice cluster-II (RC-II), marine benthic group D (MBD) and *Methanomicrobiales*: group E1 (E1), group -E1′ (E1′), group E2 (E2). The *y*-axis shows the intensity of the peaks in relative fluorescence units (RFU).

performing multiple *in silico* sequence analyses of published sequences and our clone libraries, and confirming the predicted fragment size by amplifying and T-RFLP analysing clones. Table 2 presents the identity match and predicted terminal restriction fragment (T-RF) size from the *in silico* digestion of our sequences, and Fig. 3 shows two representative profiles and their corresponding peak identifications. We were able to resolve the groups A-F described by Basiliko and colleagues (2003) and in many cases obtain better resolution. For example, group B, equivalent to the *Methanosaetaceae*, could be resolved into three groups, and group E, equivalent to the *Methanomicrobiales*, could be resolved into two groups E1 and E2, which were not entirely monophyletic and were subdivided into E1, E1′, E2 and E2′ (Fig. 2). E1′ was only found in clone libraries of deeper layers of CB, and E2′ sequences were not recovered in the libraries and correspond to cultured *Methanomicrobiales* (Fig. 2). Of other groups, the SC cluster shared the same T-RF size with a subcluster of RC-I. According to our clone libraries, both would only occur in samples from deeper layers of CB.

Assessment of the vertical community structure in duplicate core samples from both study sites is summarized in Fig. 4. The community profiles from both sites were similar

Table 2. Predicted terminal restriction fragment (T-RF) length of archaeal 16S rRNA gene sequences from clone libraries and their phylogenetic affiliation.

Phylogenetic association	T-RF length (bp)	Clone name
Methanosarcinaceae	271	MB13, MB09, CBs-a3H, CBs-b2A
Methanosaetaceae	88	MB04, MB18, CBs-a2H, CBs-b3C
	118	CBd-4610G, CBd-366G
	397	MB12
Subaqueous cluster	363	CBd-466G, CBd-461H, CBd-462E, CBd-465B
Rice cluster-I	230	MB14, CBs-b1D, CBs-b3B, CBs-c1F
	363	CBd-367A, CBd-368F, CBd-3612B, CBd-366B
Rice cluster-II	100	MB05, MB08, CBs-b2C, CBd-366D, CBd-464H, CBd-308E, CBd-361E
Methanomicrobiales group E2	233	MB16, MB19, MB02b, MB 17, MB03, CBs-e2B, CBs-c2H, CBs-c1H, CBd-364F, CBd-471E, CBd-362C, 6A8
Methanomicrobiales group E1	292	MB06, MB10, CBs-e2E, CBs-a1D, CBd-304B, CBd-472G
Methanomicrobiales group E1′	292	LH 01, LH03, CBd-463F, CBd-472C, CBd-303D
Marine benthic group D	337	CBd-472B, CBd-305F, CBd-302C

Fragment size is predicted from 3′ end for fluorescently labelled reverse primer 1100R (Hales *et al*., 1996).

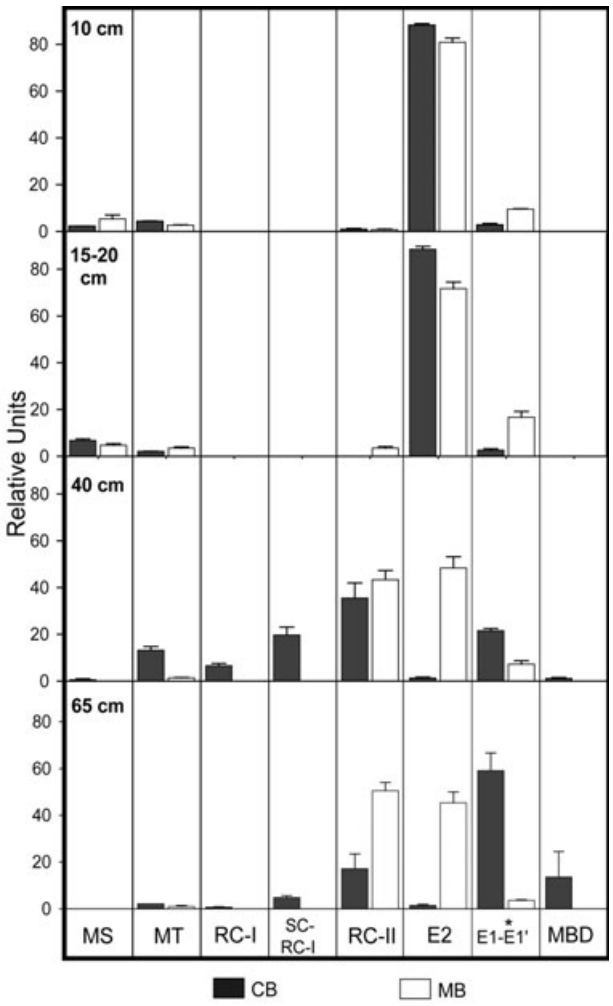


Fig. 4. Normalized T-RFLP profiles of 16S rRNA gene fragments amplified from peat samples from vertical profile of Chicago bog (CB) and McLean bog (MB). Sampling depths are indicated in each set of profiles. Peaks matching its predicted group were arranged in the same order as in their phylogenetic tree, and the *Methanosaetaceae* peaks were merged into a single group for simplicity. The archaeal groups represented by distinctive T-RFs are: *Methanosarcinaceae* (MS), *Methanosaetaceae* (MT), rice cluster-I (RC-I), rice cluster-II (RC-II), marine benthic group D (MBD) and *Methanomicrobiales*: group E1 (E1), group -E1′ (E1′), group E2 (E2). Profiles were normalized to a total of 100 units. Mean ± SD; $n = 3$. The asterisk (*) indicates E1′ sequences were only observed in CB at 40 cm and 65 cm.

at 10 and 15–20 cm. The dominant T-RF in these layers was that of group *Methanomicrobiales* E2, in agreement with its high abundance in clone libraries (data not shown). Group *Methanomicrobiales* E1 was present as a smaller fraction and was greater in MB than in CB. *Methanosarcinaceae*, *Methanosaetaceae* and RC-II T-RFs were present in upper layers at both sites as a very small fraction of the community.

The community structure at 40 and 65 cm depth clearly changed compared with the upper layers (Fig. 4), but the differences not only corresponded with depth but also between sites. Chicago bog harboured a greater number of T-RF types than MB, where MBD, RC-I and SC were consistently observed at deep layers of CB only. Group E2 decreased in dominance at both sites. However, E2 changed from being the most dominant T-RF to a very small fraction of the community (close to the technique's limit of detection) in CB; while in MB, E2 was still one of the major components in the profiles. Group E1/E1′ (Fig. 2) increased in CB, particularly at 65 cm where E1′ sequences were mainly recovered, and slightly decreased in MB. In addition, a unique *Methanosaetaceae* T-RF was present only at lower depths of CB and it had a greater abundance than the others found in higher layers particularly at 40 cm. The increase of the RC-II fraction at 40 cm depth was the only common finding between the two sites at their deeper layers.

Abundance estimation by quantitative polymerase chain reaction

We attempted to complement the T-RFLP's qualitative information with a quantitative polymerase chain reaction (qPCR) approach focused on the abundance of total *Archaea*, and groups E2 and E1. Because of the limitations imposed by the close phylogenetic relationships of group E2 and E1, we could not design primers to quantify each group separately but instead developed a single primer set that quantifies both groups together but excludes E1′- and E2′-associated sequences. For total archaeal quantification, we used universal primers that have been successfully used in qPCR in other studies (Riley-Buckley, 2001). Both sets of primers were tested for specificity and coverage and the results are summarized in Table 3.

Results of qPCR (Fig. 5A) indicated that total archaeal numbers did not strongly decline with depth in both sites (10^8–10^9 normalized gene copy number per gram of dry peat). These results, although higher in order of magnitude as they are expressed per gram of dry instead of fresh peat, were similar to those previously obtained with fluorescent *in situ* hybridization (FISH) in a Siberian acidic peatland (Kotsyurbenko *et al.*, 2004). Attempts to apply FISH to these peat samples were unsuccessful partly because of substantial autofluorescence of the peat material.

The quantification results for group E2-E1 (Fig. 5B) showed that E2 and E1 roughly constituted approximately 55% of total archaeal targets in the upper layers of both sites, with E2 as the main contributor as indicated by T-RFLP. E2-E1 target decreased to a different degree at 40 and 65 cm at both sites: in CB it decreased by roughly 2.5 orders of magnitude (~0.15% of total *Archaea*), while in MB it decreased by 1.3 orders of magnitude (~2% of total *Archaea*).

Table 3. Specificity and coverage of 16S rRNA gene primers used to quantify total *Archaea* and the *Methanomicrobiaceae* E2-E1 group.

Group/genus	Organisms tested	Reaction with	
		Archaeal primers	E2-E1 primers
Bacteria[a,b]	*Bulkholderia* sp., *Chromobacterium* sp., *Clostridium* sp., *Yersinia* sp., *Azospirillum* sp., *Magnetospirillum* sp.	–	–
Archaea[b,c]			
Thermoplasma	*T. acidophilum*	+	–
Methanococcus	*M. maripaludis*	+	–
Methanosarcina	*M. siliciae*, *M acetivorans*, *M barkeri*	+	–
Methanosaeta	*M. thermoacetophila*, *M concilii*	+	–
Methanogenium	*M. carici*	+	–
Methanocorpusculum	*M. parvum*	+	–
Methanofollis	*M. liminatants*	+	–
Methanospirillum	*M. hungatei*	+	–
E2 culture[b]	Methanogenic culture 6A	+	+
Environmental clones	CBs-c1H (group E2 clone)	+	+
	CBs-e2E (group E1 clone)	+	+
	CBs-b3C (Methanosaeta clone)	+	–

a. Bacterial isolates from MB classified by their closest phylogenetic association to bacterial groups based on their 16S rRNA gene sequence.
b. Chromosomal DNA from microorganisms cultures in PCR reaction was 0.5 ng μl^{-1}.
c. Plasmid DNA for environmental clones was used in 10^6 copies μl^{-1}.

Discussion

McLean bog and CB are typical of acidic ombrotrophic (ombro- = rain fed; Crum, 1992) bogs with low mineral content (Blodau, 2002). Upper layer at both sites shared similar physicochemical characteristics, but their deeper layers exhibited significant differences, particularly pH (Table 1) and peat composition. In MB and CB, methanogenic activity in lower layers using endogenous substrates was 1–1.5 orders of magnitude lower and there was little if any initial stimulation by addition of methanogenic substrates, indicating that the methanogenic populations were at low levels and had low activity.

Hydrogenotrophic and aceticlastic, i.e. H_2/CO_2 and acetate based, methanogenesis are frequently observed in wetlands (Whalen, 2005). In acidic ombrotrophic bogs, the former has been commonly reported as the predominant process (Williams and Crawford, 1984; Lansdown *et al*., 1992; Avery *et al*., 1999; 2003; Chasar *et al*., 2000; Hornibrook *et al*., 2000; Duddleston *et al*., 2002; Nakagawa *et al*., 2002; Horn *et al*., 2003; Galand *et al*., 2005), although the latter has recently been shown as the main

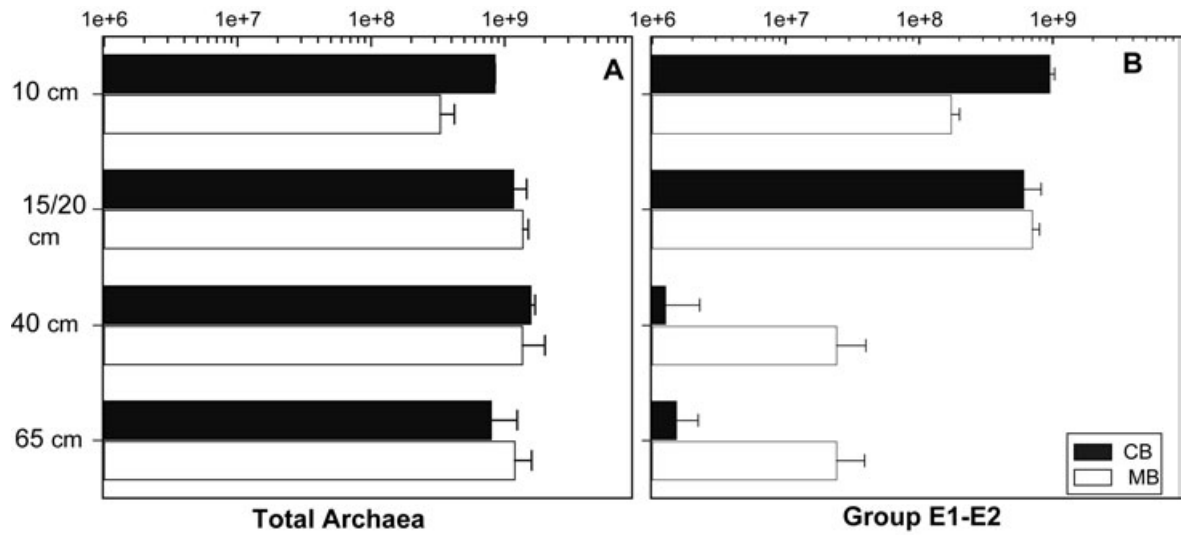


Fig. 5. Real-time qPCR quantification of 16S rRNA gene copy number of total *Archaea* (A) and the E2-E1 group (B) in DNA extracts from peat samples from the vertical profile of Chicago bog (CB: black bars) and McLean bog (MB: white bars). The *x*-axis represents the number of gene copies normalized by two rRNA operon copies and expressed in logarithmic scale; *y*-axis indicates the depth at which the sample was taken.

contributor in a pH 4.8 Siberian bog (Kotsyurbenko *et al.*, 2004). Our incubation results showed that H_2/CO_2-amended samples produced significantly more CH_4 than those amended with acetate, indicating a greater potential for this pathway. The most methanogenically active layers, the upper 20 cm, just below the water table level, had a minimal lag time and rates increased within a few days after the incubations begun (Fig. 1A and B). This supports the existence of active and rapidly responding hydrogenotrophic methanogen populations at upper layers. The long lags demonstrated by samples from lower layers incubated with H_2/CO_2 indicated that populations of hydrogenotrophic methanogens were low and that perhaps only a specific subpopulation could be stimulated by addition of substrate.

On the other hand, our results for potential aceticlastic methanogenesis showed stimulation after a 20-day lag period in both upper layers of MB's and CB's 15 cm (Fig. 1). This indicates that the acetate-utilizing populations were near saturation for acetate, which we have measured as < 50 μM (S. Bräuer, unpublished) and are slow to respond to substrate addition. Although aceticlastic methanogens in MB can be stimulated with low doses of acetate (Bräuer *et al.*, 2004; our results), and Kotsyurbenko and colleagues (2004) demonstrated a case in which this pathway is responsible for twice as much methanogenesis as the hydrogenotrophic one, typical of neutral-pH habitats, the contribution of aceticlastic methanogenesis in acidic northern peatlands is not yet clear.

The 16S rRNA gene clone library of Basiliko and colleagues (2003) for MB as well as those presented here for CB demonstrated the presence of members of the *Methanomicrobiales*, known to use H_2/CO_2, as well as the *Methanosarcinaceae* and *Methanosaetaceae*, which are known to be aceticlastic. Additionally, other euryarchaeal groups such as RC-I, RC-II, MBD and SC were observed at our sites. These RC-I and RC-II have been found in different ecosystems with active methanogenesis, such as acidic peatlands, rice paddies and river or lake sediments (Chin *et al.*, 1999; Galand *et al.*, 2002; Glissman *et al.*, 2004). The MBD has been recovered from marine (Vetriani *et al.*, 1999) and lake sediments (Jurgens *et al.*, 2000; Nusslein *et al.*, 2001), and it is associated with RC-III and *Thermoplasma* groups (Kemnitz *et al.*, 2005) found in anoxic soils. In this study we are proposing a new group of environmental phylotypes under the SC denomination. Members of this group were found at deep levels of CB, and were only associated with other sequences reported from submerged or deep environments (Stein *et al.*, 2002; Schrenk *et al.*, 2003; Nedelkova, 2005) and soil contaminated with material from subsurface environments such as petroleum (Kasai *et al.*, 2005). We speculate that SC members are specialized for survival in anaerobic, commonly submerged and highly reduced environments like deep layers of peat, lake sediments in deep sections or deep wells. More studies are required to assess the distribution of SC and their role in these environments.

The results of 16S rRNA gene sequencing coupled to T-RFLP analyses allowed minimization of ambiguous peak identification. The T-RFLP parameters we developed are useful for studies on freshwater methanogenic populations, particularly in peatland ecosystems. Our results showed that the T-RFLP profiles of the upper peat layers at both sites were similar both in terms of community structure and in terms of physicochemical characteristics (Table 1 and Fig. 4). *Methanomicrobiales*-related phylotypes were resolved into three groups: E1, E1′ and E2 by sequence clustering. E2 has also been called R10 cluster (Edwards *et al.*, 1998) and fen cluster (Galand *et al.*, 2002) in other studies. E1′ was only detected in clone libraries from deeper samples in CB (data not shown). *Methanospirillum hungatei* was the closest cultured organism to the E1 and E2 groups, but all three groups (E1, E2 and E1′) represent novel methanogens whose physiology has yet to be elucidated.

Group E2 dominated T-RFLP profiles from the upper methanogenically active layers of both CB and MB, with smaller contributions from E1, similar to distributions in 16S rRNA gene clone libraries. Sequences in the E2 group have been found in an H_2/CO_2 enrichment (Sizova *et al.*, 2003), and we have cultured a member of this group (6A in Fig. 2) on H_2/CO_2 (Bräuer *et al.*, 2006). *Methanosaetaceae* and *Methanosarcinaceae* represented less than 10% of the total peak height in T-RFLP profiles, consistent with a modest contribution of methanogenesis from acetate to total methanogenesis. In studies of a neutral-pH fen, these two groups made up $> 50\%$ of the peak height (C. Sun, unpublished) indicating that the PCR primers and conditions were not biased against these groups.

The numerical dominance of hydrogenotrophic methanogens in the upper layers suggested by T-RFLP was confirmed with our real-time qPCR 16S rRNA gene target quantification, which estimated that the abundance of groups E2 and E1 together (although E1 contribution was small, particularly in CB samples) roughly accounted for ~55% of total archaeal gene targets in the upper layers, with their populations reaching a size on the order of 10^8 cells per gram of dry peat. These results clearly indicate the importance of group E2 for CH_4 production by CO_2 reduction in these *Sphagnum*-dominated sites. The E2 group is not unique to our study sites in the northeast USA, but instead has a widespread distribution in peatlands in the northern hemisphere. Phylotypes clustering within group E2 have been found in bogs from Germany (Horn *et al.*, 2003), Finland (Galand *et al.*, 2003), the UK (Edwards *et al.*, 1998) and Canada (J. Yavitt, unpub-

lished), making up a significant fraction of clones in libraries or peak heights in T-RFLP analysis. An exception appears to be a pH 4.8 Siberian peat sample (Kotsyurbenko *et al*., 2004) in which E2 was only a small proportion of the methanogenic community. We speculate that this group is a significant contributor to methanogenesis in highly acidic peatlands. This hypothesis is in agreement with the significant presence of the 'fen cluster' in Finnish peatlands (Galand *et al*., 2002; 2005; Juottonen *et al*., 2005) whose *mcrA* sequences are phylogenetically associated with the E2 culture (96% similarity; Bräuer *et al*., 2006). The population size and activity of group E2 at additional sites is required in order to confirm this findings.

In the vertical profiles, group E2 decreased in proportion of methanogens in T-RFLP (Fig. 4) and in numbers (Fig. 5) at both sites in samples from 40 and 65 cm. These deep samples also showed low rates of endogenous and substrate-stimulated methanogenesis. In MB, E2 still represented about half of the total peak height, whereas it was barely detectable in the more neutral CB deep samples, consistent with its being associated with low-pH habitats. Group E2's preference for acidic conditions is supported by the observations of Bräuer and colleagues (2004), where the pH optimum for peat incubations from MB was 4.8 in H_2/CO_2-amended treatments and that a shift to 5.5 or 6 strongly decreased CH_4 production.

Other significant changes in the community structure at depths below 40 cm were related to an increase in RC-II at both sites, and the presence of RC-I, MBD, SC and increase of E1 in CB. Rice cluster-II became an important fraction of the euryarchaeal community at both sites regardless of the pH differences, suggesting it has members that thrive in a broad range of pH values; this is supported by the fact that its sequences have been retrieved from acidic as well as neutral sites (Chin *et al*., 1999; Basiliko *et al*., 2003; Kemnitz *et al*., 2004). No isolated representative of RC, MBD or SC groups have been obtained up until now; although RC-I has been highly enriched with H_2/CO_2 from peatland and rice paddy soil samples with some initial physiological and genomic characterization (Lueders *et al*., 2001; Sizova *et al*., 2003; Erkel *et al*., 2005). It is interesting that the samples from CB lower levels, despite having low rates of methanogenesis, show considerably higher diversity than the upper levels, perhaps remnants of methanogenic populations present from a neutral-pH fen.

Experimental procedures

Study sites and sampling

McLean bog and CB are located within 40 km of Ithaca, New York (42°30′N, 76°30′W) in the northeast USA, and both sites were initially described by Osvald (1970). McLean bog is an ombrotrophic kettle hole bog approximately 70 m across (0.04 km^2), the peat is 8 m deep, and the vegetation is dominated by *Sphagnum* moss (*S. angustifolium* and *S. magellanicum*). Vascular plants including ericaceous shrubs (*Chamaedaphne calyculata*, and *Vaccinium corymbosum*), three-way sedge (*Dolichium* spp.) and pitcher plants (*Sarracenia purpurea*) are also present in significant numbers. Chicago bog is an oligotrophic floating bog of approximately 0.05 km^2, and is associated with a pond on its east side. Its current surface is sphagnum dominated, and its earlier stages of peat development were sedge derived (Dettling, 2005). The *Sphagnum* species covering this bog (e.g. *S. fuscum*, *S. magellenicum*) are responsible for its hummocky surface; and the drier hummocks support ericaceous shrubs (*C. calyculata*) which are dominant in the site.

Sampling was performed on 28 June in CB and 4 October for MB in 2004. Duplicate cores were taken at both sites using a polyvinyl chloride (PVC) coring device with airtight seals. Sample points along vertical profile were determined by considering the physical characteristics of the peat in a parallel core obtained with a Russian style peat corer device. Airtight sealed cores were immediately transported to the laboratory and sampled inside an anaerobic glove box (Coy Laboratory Products, USA) with an atmosphere of N_2 and 2–4% H_2. Peat samples from each of the two cores taken from each site were individually assayed. As the replicate cores had similar results, only those from one core from each site are presented.

CH_4 production

Peat slurry incubations with or without substrate additions were performed as described by Bräuer and colleagues (2004). Briefly, inside an anaerobic glove box, 1 g of peat was added to anaerobic deionized water to a volume of 10 ml in 18×150 mm crimp-top tubes. The tubes were sealed with butyl rubber stoppers and were flushed with sterile O_2-scrubbed N_2/CO_2 (70%/30%, Mixed Gas Industries, USA). Anaerobic stock solutions were added as indicated to the following final concentrations: sodium acetate (1 mM), rifampicin (10 mg l^{-1}). Sterile O_2-scrubbed H_2/CO_2 (80%/20%, 70.7 kPa, Mixed Gas Industries, USA) was added to appropriate tubes. Incubations were performed at room temperature (~23°C) in the dark, under static conditions for samples with no addition or 1 mM acetate, and shaking (225 r.p.m.) for samples with rifampicin, or rifampicin + H_2/CO_2. Presented data represent the averages of triplicate samples.

Chemical analyses

Headspace gas was analysed for CH_4 using a Perkin Elmer 3920B gas chromatography column with a flame ionization detector (Phoenix Equipment, USA). Peat slurries were vortexed for 30 s before headspace analysis. Dry weight and pH of triplicate peat samples were determined as described elsewhere (Bräuer *et al*., 2004) within 24 h after sampling.

DNA extraction and PCR amplification

Triplicate samples from each depth in the peat core were extracted with the Power Soil™ DNA kit (MoBio, USA) using

the manufacturer's protocol with some slight modifications. Briefly, 0.5 g of peat was mixed with beads and disrupting solution, and after adding the C1 solution we also added 50 μl of a sterile 200 mM $AlNH_4(SO_4)_2$ solution to avoid humic acid co-purification (Braid *et al*., 2003). The mixture was subjected to 1 min 20 s of bead beating at maximum speed (MiniBeadbeater™, Biospect Products, USA). The quality of recovered DNA solution was examined by 2% agarose gel electrophoresis and spectrophotometer readings at 230 and 260 nm; minimal DNA shearing and $A_{260/230}$ ratios close to 2 were observed in almost all samples.

A fragment from positions 1–1100 (*Escherichia coli* numbering) of the 16S rRNA gene was amplified using the archaeal-specific 1AF (5′-TCY GKT TGA TCC YGS CRG AG-3′)-1100R (5′-TGG GTC TCG CTC GTT G-3′) set of primers (Hales *et al*., 1996). The PCR mixture contained the following components at its reactants concentrations per μl: 1× Taq buffer with 1.5 mM $MgCl_2$ (Eppendorf, USA), 0.2 mM deoxynucleotide triphosphates (dNTP), 0.25 μM forward and reverse primers, 1.2 U of *Taq* Polymerase (Eppendorf, USA), 0.2 μg of bovine serum albumin (BSA) and 0.1–0.3 ng of extracted DNA. The PCR conditions were as described by Hales and colleagues (1996) with 25 amplification cycles. Amplification products were examined by electrophoresis on 1% agarose gels for size verification.

Cloning, sequencing and phylogenetic analysis

16S rRNA gene clone libraries were constructed as described by Basiliko and colleagues (2003). Six clone libraries were constructed for samples from 15 cm, from 40 and 65 cm depth in CB. Using the TA Cloning kit® (Invitrogen, USA) and m13 primer screening, 60 clones per library were selected for restriction analysis with HaeIII and HhaI enzymes (New England Biolabs, USA). Clones displaying unique restriction patterns were sequenced with an ABI 3730 automated sequencer (Bio Resources Center, Cornell University). Sequences were compared against the GenBank database (Benson *et al*., 2004) in order to ensure that newly reported relatives were included in our database.

Phylogenetic analyses of the sequences were performed using the ARB software (Ludwig *et al*., 2004) with the latest 16S rRNA sequences database release (released August 2003; http://www.arb-home.de) and an 'archaeal database' with 2500 complete and partial archaeal sequences (Jurgens, 2002). Phylogenetic placement was performed using the quartet PUZZLE method implemented in ARB in comparison with reference archaeal sequences. A nucleotide base frequency filter that included positions with more than 50% invariance (1020 valid columns) was used to avoid possible treeing artefacts. Sequences were added to the tree reference sequence tree using the ARB parsimony tool without altering global tree topology. Tree topology was confirmed using maximum likelihood, neighbour joining and FITCH methods (as implemented in the ARB package).

The sequences of the 16S rRNA gene clones obtained in this study have been deposited in the GenBank nucleotide sequence database under the Accession No. DQ301878 to DQ301915.

T-RFLP analysis

We used the PCR primers and conditions described above. The 1100R reverse primer was fluorescently labelled on its 5′ end with Carboxifluorescein (5′-/6-FAM). Terminal restriction fragment length polymorphism analysis was performed as described elsewhere (Marsh, 1999) with some modifications. Briefly, 30 μl of triplicate PCR reaction per sample was pooled and purified with the Quick Step™2 PCR Purification Kit (Edge Biosystems, USA), and 70 ng was digested with a mix of HhaI (15 U) and Sau96I (10 U) enzymes (New England Biolabs, USA) for 3 h at 37°C. Digested DNA was purified with the Performa® DTRV3 96-Well Short Plate Kit (Edge Biosystems, USA). Purified products were concentrated in a vacuum centrifuge, and then resuspended with a mix of Hi Di-Formamide (Applied Biosystems, USA) and Gene Scan 500-Liz marker (12 μl ml^{-1}; Applied Biosystems, USA). Fragments were resolved with an Applied BioSystems 3730xl DNA Analyser (Bio Resources Center, Cornell University).

Terminal restriction fragment sequence length, peak height and area were determined using the GeneScan Analysis Software (Applied Biosystems, 2000). GeneScan's results containing peak size, height and area were exported for profile standardization of each sample. Using a Java-based routine, the relative fluorescent units (RFU) of peaks with size 50–500 bases were added by height or area and normalized to 100 total RFU. The standardization step minimized peak height and area variation from sample to sample and did not affect the profiles' morphologies. Standardized profiles from samples taken in triplicate at each depth were averaged by their correspondent peak heights, and standard deviations were calculated.

Real-time quantitative PCR

Primer selection for total *Archaea*, and primer design for group E2-E1, was performed considering previously described optimal conditions (Suzuki *et al*., 2000; Nadkarni *et al*., 2002) and primer efficiency and coverage. For total *Archaea*, the Arch 967 F primer 5′-AAT TGG CGG GGG AGC AC-3′ (Amann *et al*., 1990; Riley-Buckley, 2001) combined with ArcH-1060R – the reverse complement of A1040F (Reysenbach and Pace, 1995) – had a broad euryarchaeal and crenarchaeal coverage with no matches for bacterial or eukaryotic sequences as reviewed for A1040F(Baker *et al*., 2003). For group E2-E1 joint quantification, we used the AgE372 (5′-ACT CCG AGT GCC CGT WAA ATC-3′) and AgE540aR (5′-AGT AAT AGT GGC CAC CAC TCG AGC-3′) set of primers, designed in this study with the primer-design and probe-match tool of ARB software. Predicted coverage and specificity analyses used the latest ARB database, the 'Archaeal database' (Jurgens, 2002) and Ribosomal Database Project II (Cole *et al*., 2003), confirming that all the archaeal phylotypes retrieved from our study sites could be amplified by the archaeal primers and that the E1-E2 primers only amplified members from this group (data not shown). Both primers were challenged with unspecific and specific targets as shown in Table 3.

Primer concentration was optimized using iTaq SYBR Green Supermix with ROX (Bio-Rad, USA). Polymerase

chain reactions (30 μl) contained 1× Supermix, 0.25 and 0.2 μM forward and reverse primers (for total *Archaea* and E2-E1 respectively), 0.1 g l^{-1} BSA and 3 μl of extracted DNA. Amplifications were performed on duplicate samples at two dilutions (1:5 and 1:10) using an ABI Prism® 7000 Sequence Detector (Applied Biosystems, USA) with the following settings: 2 min at 50°C, 10 min at 95°C, and 38 cycles of 15 s at 95°C with 1 min at 60°C. This was followed by a dissociation protocol to check for proper dissociation profiles, otherwise reactions were rejected. Plasmid DNA external standards with 10^8–10^2 target copies per microlitre were constructed for quantification as described by Fey and colleagues (2004).

Real-time PCR results were normalized to per cent peat dry weight at each depth and by the arbitrary value of two rRNA gene operons per genome, the closest integer to the currently reported archaeal average in the Ribosomal RNA Operon Copy Number Database (Kaplenbach *et al.*, 2001).

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